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An increase in dendritic spine density on hippocampal CA1 pyramidal cells following spatial learning in adult rats suggests the formation of new synapses

(hippocampus/plasticity/confocal microscopy/complex environment)

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ABSTRACT **The search for cellular correlates of learning is a major challenge in neurobiology. The hippocampal formation is important for learning spatial relations. A possible long-lasting consequence of such spatial learning is alteration of the size, shape, or number of excitatory synapses. The dendritic spine density is a good index for the number of hippocampal excitatory synapses. By using laser-scanning confocal microscopy, we observed a significantly increased spine density in CA1 basal dendrites of spatially trained rats when compared to nontrained controls. With unchanged dendritic length, the higher spine density reflects an increased number of excitatory synapses per neuron associated with spatial learning.**

The hippocampal formation is closely related to spatial learning. This conclusion is based upon the presence of cells signaling the position of the animal in space and the interference with the ability to learn a spatial environment following mechanical or chemical inactivation of the hippocampus and neighboring cortex (1–4). Many of the hippocampal synapses have plastic properties, which may play a role in the learning process (5–7). Since learning effects are long-lasting, structural changes of hippocampal synapses are possible correlates to spatial learning. Among possible changes, the alteration of the size, shape, or number of excitatory synapses is among the most likely ones. Because virtually all excitatory synapses on hippocampal pyramidal cells contact dendritic spines (8), the number and distribution of these structures may be taken as an index of synaptic changes. Training in a complex environment causes spatial learning (9–12). Whereas exposure to an enriched environment gives various structural changes in the visual cortex, there are few reports on such effects in the hippocampus (13). Using two-dimensional electron microscopy, Altschuler (14) found an increased number of synapses on CA3 cells in young rats after training in an enriched environment. In the absence of information on dendritic length, which is environmentally modifiable (15), it is uncertain how such changes translate to spine density. Further, the large increase in number of boutons (82%), observed during a developmental period with rapid changes of spine density (16), need not be related to learning, but to normal development. Wenzel *et al.* (17) reported increased CA1 spine density after training, but the selected brightness discrimination learning probably does not depend upon the hippocampal formation (18). Further, the spine density for the control and experimental material reported by Wenzel *et al.* (17) were both within the normal spine density range given by Andersen *et al.* for guinea pigs (19).

On this background, we chose a hippocampus-dependent task. We tested whether or not spatial training of rats can give changes in dendritic spine density in the CA1 field of the hippocampus. To avoid interference with developmental processes, we used adult animals.

MATERIALS AND METHODS

Environment. Adult male rats (250–460 g) were kept together in a large (2.5 × 2.0 × 1.7 m) cage with up to five floors mounted at various positions and connected with narrow ladders (Fig. 1*A*). Items expected to generate exploration (wooden blocks, freshly cut wood chips, branches, fresh leaves, plastic containers, paper bags) were distributed on the floors. Water and food bowls were hidden. All floors, ladders, and the position of water and food were changed between sessions. The rats were exposed to this environment for 4 hr/day for 18 days (behavioral study, $n = 7$) or 14–30 days (morphological study, $n = 13$). Between the exposures, the rats were housed in groups of 6 or less in transparent cages (59 × 39 × 20 cm). The activity of the animals (number of floors visited, area covered, latency to new floors, latency to all floors) was monitored by two independent observers during the first hour of each training session. Control rats were either housed in pairs ($n = 8$) in transparent cages (37 × 22 × 18 cm) or individually ($n = 7$) in opaque cages (33 × 20 × 18 cm). All rats were food-deprived for 19 of the preceding 24 hr.

Water Maze. In a subset of rats ($n = 7$, trained; $n = 10$, paired; $n = 9$, isolated) spatial learning was tested in a water maze (20), a circular pool (diameter 198 cm), filled to a depth of 40 cm with water to which milk was added. A platform was positioned in one of four possible places, halfway between the center and periphery. Each rat was assigned a platform position that was maintained throughout the experiment. The rats were trained in two daily sessions 4 hr apart. Each session consisted of four trials 40 s apart. The rat swam from randomized start positions. If the rat failed to find the platform, it was guided to it after 120 s. All rats were left on the platform for 30 s. The position of the rats was monitored by a vertically mounted camera and stored at 10 Hz. When all rats had learned to swim directly toward the platform (after session 9), the environment was restructured, curtains were drawn to form a square around the pool at a distance of at least 1 m, and cues were attached. After the environmental change, the platform was located in a new, fixed position on all trials.

Staining and Preparation. Animals assigned to the morphological study ($n = 26$) were exposed to one of three environments ($n = 11$, trained rats; $n = 8$, paired rats; $n = 7$, isolated rats) but were not tested in the water maze. The animals were deeply anesthetized with halothane and decapitated by a colleague in the laboratory, so that the investigators remained ignorant of the behavioral history of the animals. The brain was

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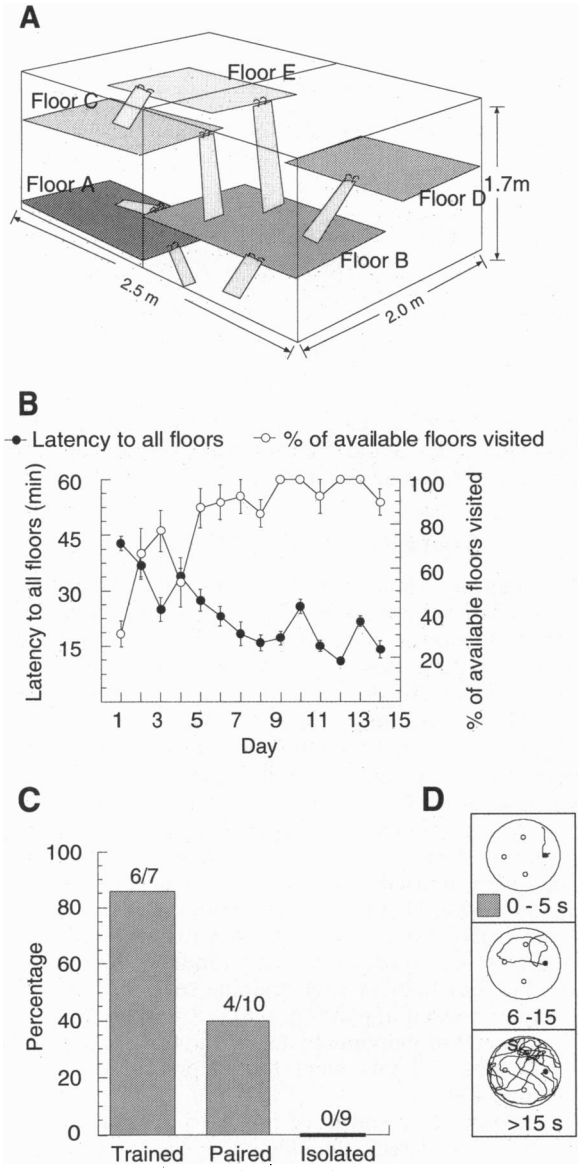

FIG. 1. Spatial training in the complex environment and testing of the acquired learning in a water maze. (*A*) Diagram of the complex environment. (*B*) Exploration intensity during the first hour of the total 4-hr exposure in the complex environment, expressed as the mean latency for all animals ($n = 11$) to reach floors (●) and as the percentage of available floors visited during the first hour (○). (*C*) Percentage of rats belonging to the best category (0–5 s, in *D*) on trial 2 in a new water maze environment. (*D*) Swim paths of three rats, in the good (0–5 s), medium (6–15 s), and poor learning categories (>15 s).

taken out and the hippocampus was dissected free in cold (1–3°C) artificial cerebrospinal fluid (in millimolar: NaCl 124, $NaHPO_4$ 1.25, KCl 3, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10), and 400-μm-thick slices were cut with a vibratome. The slices were placed in the interface between artificial cerebrospinal fluid from below and a humidified gas mixture (95% O_2/5% CO_2) above. The inner part of the gas chamber was kept at 32 ± 0.5°C. Fiber-containing electrodes were filled with Lucifer yellow (4% in 0.2 M LiCl; resistance, 75–200 MΩ). After 1 hr in the bath, CA1 cells were penetrated and the dye was injected with hyperpolarizing pulses (1.5 nA). Adequate spine staining required the cells to show action potentials above 80 mV and a duration of <2 ms. After filling, the slices were fixed in paraformaldehyde (4%), dehydrated in an alcohol series (75%, 85%, 95%, 96%, and 100%), cleared in methyl salicylate, and coverslipped in DPX (BDH).

Morphological Analysis. Only cells with an extensively filled dendritic tree without obvious truncations were analyzed in a laser-scanning confocal microscope (Phoibos 1000 Sarastro, equipped with the software package Image Space, Molecular Dynamics). The data were stored on optical disks for later analysis. A 40× objective (numerical aperture, 1.3) was used for an overview of cells by making projections from section series (image size, 512 × 512 pixels; pixel size, 0.5 μm; step size, 3.0 μm; 40–80 sections). Dendritic length and branching pattern were calculated with the programs PDENLEN and NEUREC (unpublished) provided by T. Blackstad (Department of Anatomy, University of Oslo). For the spine counts, four or five dendritic segments were picked from the middle portion of the basal dendrites. For detailed analysis, sections were scanned with a 100× objective (numerical aperture, 1.3) (image size, 256 × 256 pixels; pixel size, 0.1 μm; step size, 0.1 μm; 20–70 sections). The criteria for spine detection followed those given in ref. 21. The data analysis was made blind with a subset of spines studied by all three investigators. Student's *t* test for comparison gave no significant difference between either pair of the three investigators' analyses.

RESULTS AND DISCUSSION

Spatial Learning. We first wanted to ascertain that the training caused an increased spatial learning ability. During the spatial training period in the complex environment, the animals gradually increased their activity both with regard to the numbers of visited floors and the total area covered (Fig. 1*B*).

We tested the effect of the spatial training by recording the animals' performance in a Morris water maze. The spatially trained animals acquired the standard water maze task faster than both control groups [$F(2, 23) = 4.16$, $P < 0.03$]. However, after six or seven training sessions (four trials each), animals from all groups found the hidden platform equally fast and showed similar precision in a test without the platform. At the end of the acquisition period, the animals were tested for their ability to solve a new task of the same nature. A new environment was made by fastening cues to curtains forming a square around the water maze at a distance of at least 1 m. The animals trained in the complex environment acquired the new platform position faster and swam more directly to the new position on the second trial than the nontrained animals (Fig. 1 *C* and *D*, $P < 0.05$, one-sided Wilcoxon test). The latency to find the platform on the second trial (mean ± SEM) was 4.7 ± 0.6 s in the trained group against 8.9 ± 1.4 s in the paired and 25.2 ± 9.3 s in the isolated groups. The mean number of pool quadrants entered before reaching the platform was 1.0 ± 0, 2.6 ± 0.6, and 5.3 ± 1.6 for the same groups, respectively. Thus, the spatial training in the complex environment gave an improved acquisition rate of the water maze tasks, even though the three groups reached similar performance levels with repeated training.

Spine Density. Another group of animals was similarly trained in the complex environment for 14–30 days. After training, slices were taken from these rats and from two groups of control animals as before. With our technique based on laser-scanning confocal microscopy of cells injected with Lucifer yellow (21), a large number of spines were counted from various portions of identified neurons. Satisfactory large and representative samples, coupled to a high rate of spine detection, make the technique useful for measurement of spine density changes following experimental interference. Fig. 2 shows examples of basal dendritic segments taken from two different CA1 cells to illustrate the appearance of spines and the variability of the spine density. Cells taken from rats after intense spatial training had a spine

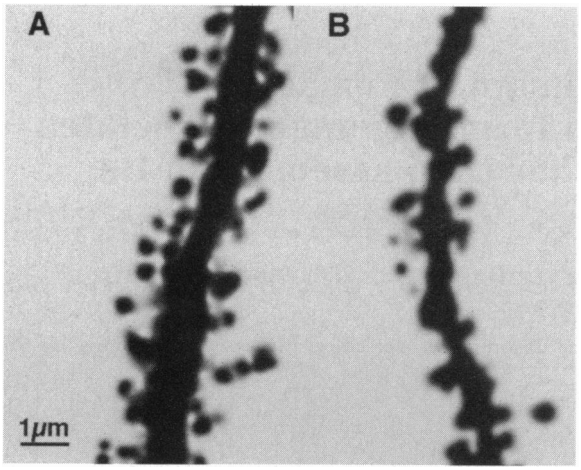

FIG. 2. Examples of parts of two basal dendritic segments to illustrate the variation of spine density. The spine densities were 2.71 spines per μm (trained rat, *A*) and 1.75 spines per μm (isolated rat, *B*).

density of 1.96 ± 0.09 spines per μm (mean ± SEM; n = 11 rats, 23 cells, 137 dendritic segments, and 7460 spines). The spine density of the paired control group was 1.77 ± 0.09 (n = 8 rats, 13 cells, 74 segments, and 3720 spines) and measured 1.78 ± 0.06 spines per μm in the isolated control group (n = 7 rats, 10 cells, 68 segments, and 3460 spines). The distribution of spine densities is shown in Fig. 3. Because the values from the two control groups were similar, these were combined for the statistical analysis. The difference in mean spine density between the spatially trained and the pooled control groups was statistically significant [Wilcoxon, one-sided test, $W(11, 15) = 181$, $P < 0.05$]. The total dendritic length and the degree of dendritic branching were similar in the three animal groups. Thus, the enhanced spine density in the trained group is not due to an altered size of the sampled neurons.

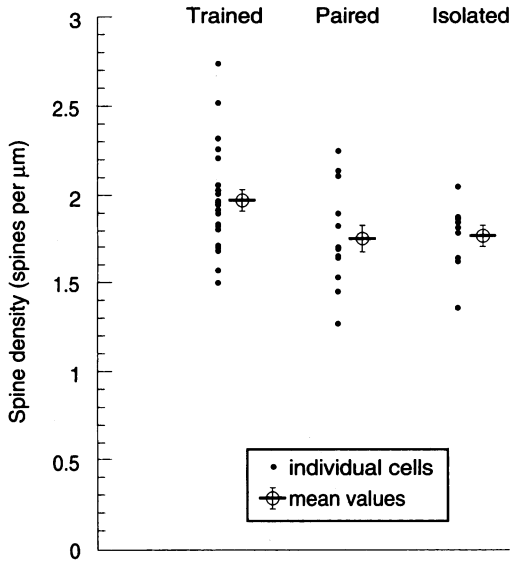

FIG. 3. Distribution of spine densities (spines per μm; filled dots) in the trained, paired, and isolated groups. Each dot represents the mean of all segments from one cell. Circles and horizontal lines give the means and the vertical lines give the SEM of the spine density in these cells in the three groups.

There was an appreciable variability between different dendritic segments from the same cell and from different cells and rats, with figures ranging from 0.90 to 2.99 spines per μm (cell means ranging from 1.26 to 2.73 spines per μm). This fact underlines the need to obtain a large sample of segments to get a representative sample for the analysis. The density of spines was considerably higher than found with Golgi impregnated material (22).

In conclusion, spatial training of adult rats by exposing them to a spatially challenging environment is associated with an increased spine density on CA1 pyramidal cells. The trained animals also showed increased learning ability as signaled by a faster acquisition in a water maze task. Because the total dendritic length was unchanged, the increased spine density means a higher number of synapses per cell, and thus an altered connectivity, as a result of spatial learning. These findings demonstrate that behavioral training can induce structural change in the hippocampal cortex of adult rats.

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